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The Ancestral Carnivore Karyotype (2*n* = 38) Lives Today in Ringtails

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Abstract

Chromosome painting was used to investigate the conservation of high-resolution longitudinal 4′,6-diamidino-2-phenylindole (DAPI)/G bands in Carnivore chromosomes. Cat (*Felis catus*) and raccoon dog (*Nyctereutes procyonoides*) painting probes were hybridized to the ringtail (*Bassaricus astutus*), dwarf mongoose (*Helogale parvula*), and Malagasy civet (*Fossa fossa*) to identify homologous chromosome elements. The patterns of chromosome segment homology among Carnivore species allowed us to reconstruct and propose the disposition of a high-resolution banded ancestral carnivore karyotype (ACK). Three bi-armed chromosomes consistently found among Caniformia species are represented as 6 homologous acrocentric chromosomes among Feliformia species of Carnivora. However, reexamination of the most basal of Feliformia species, the African palm civet Nandinia, revealed the presence of the 3 heretofore Caniformia bi-armed chromosomes. Because these 3 bi-armed chromosomes are found in both Caniformia and Feliformia lineages, they are presumed ancestral for all Carnivora, suggesting that the ACK chromosome number would be 38, rather than the previously supposed 42. Banded chromosomes of the ACK are used to evaluate the consistency between recently determined molecular phylogenetic relationships and postulated cytogenetic dynamics in the same Carnivore species.

Chromosomes of numerous species of Carnivores have been analyzed extensively with comparative conventional banding and chromosome painting. As a result, chromosomes of the ancestral carnivore karyotype (ACK) are arguably more thoroughly explored than for other order of mammals (Murphy et al. 2001). The task of defining the ACK has been facilitated by 2 serendipitous features of this group. The chromosomes in species of all but 2 families (Ursidae and Canidae) of Carnivores are highly conserved (Wurster-Hill and Gray 1975; Wurster-Hill and Centerwall 1982; Nash et al. 2001). Secondly, the order split into the Caniformia and Feliformia suborder branches very early in the Carnivore radiation (Flynn et al. 2005). Therefore, each branch acts as an outgroup for the other branch. Chromosomes identical by G-banding and zoo-fluorescence-in-situ-hybridization (Zoo-FISH), found in both branches, likely trace their origins to a common ancestor.

The number, relative size, and morphology of each chromosome of the ACK have been described previously (Murphy et al. 2001; Nash et al. 2001). Other authors are increasingly using this karyotype to evaluate Carnivora chromosome evolution and phylogeny (Nie et al. 2002; Tian et al. 2004; Perelman et al. 2005). Although cat chromosome analogues of the ACK form the basis for these comparisons, not all cat chromosomes are ancestral. Here we propose a 4′,6-diamidino-2-phenylindole (DAPI)-/G-banded version of the chromosomes of the ACK. We have chosen examples of ancestral chromosomes from several species. The chromosomes chosen from each species are considered ancestral when they occur among multiple species of both Caniformia and Feliformia lineages. To validate the proposed ACK, we compared chromosome segment homology using chromosome painting across the genomes of the domestic cat *Felis catus* (FCA), ringtail *Bassaricus astutus* (BAS), dwarf mongoose *Helogale parvula* (HPA), and Malagasy civet *Fossa fossa* (FFO). These species represent 3 families from the Feliformia, Felidae, Viverridae, Herpestidae, and one family from the Caniformia, Procyonidae. As a further check of the accuracy of the proposed banded ACK chromosomes, they are compared with the more

rearranged chromosomes of the giant panda (*Ailuropoda melanoleuca*, AME). The DAPI/G band for band homology seen in the chromosome of these modern species and the giant panda (AME) karyotype suggest that they have been conserved throughout their ancestral descent.

In the same way that a normal banded human karyotype is an essential reference for interpreting the detailed sequence of structural chromosome changes in cancer cytogenetics, a banded ACK provides the best reference for analyzing chromosome evolution in the Carnivore radiation. Chromosome painting supported by extended and well-banded chromosomes visually illuminates the subtle details as well as the more obvious features of these rearrangements. Several examples are used to illustrate the power of this approach.

Although the African palm civet *Nandinia binotata* (NBI) has traditionally been placed in the Viverridae, recent molecular phylogeny studies show it to be basal to all other Feliformia groups (Flynn and Nedbal 1998; Gaubert and Vernon 2003; Yu et al. 2004; Flynn et al. 2005). Mckenna and Bell (1997) place NBI as a monotypic Feliformia species in its own family Nandiniidae. Here we show that *Nandinia* retains 3 "marker" chromosomes that are typical of Caniformia species, but absent in other Feliformia species. The most parsimonious explanation for this observation requires that these 3 bi-armed chromosomes be considered "ancestral," in contrast to the previous ACK proposition ($2n = 42$). We describe and illustrate here a revised ACK karyotype ($2n = 38$) (see Figure 2) and reconstruct the parsimony logic that supports this inference.

Materials and Methods

Metaphase Preparations

Metaphase chromosome preparations were obtained from primary skin fibroblast cultures from the domestic cat (FCA, cell line FCA-215), ringtail (BAS 1), dwarf mongoose (HPA 1), and Malagasy civet (FFO 1). The cell lines used for obtaining chromosome metaphase spreads were kindly supplied by Dr Stephen O'Brien, PhD (Laboratory of Genomic Diversity, Frederick, MD). DAPI-, G-, and C-banding patterns were obtained following the methods of Seabright (1971), Sumner (1972), Lin et al. (1977), Modi et al. (1987), and Nash et al. (2001).

Cross-Species Hybridizations

Chromosome-specific painting probes for the domestic cat (FCA) and raccoon dog (*Nectereutes procyonides*, NPR) were made by degenerate oligonucleotide polymerase chain reaction (DOP-PCR) amplification of flow-sorted chromosomes as previously described (Wienberg et al. 1997; Nash et al. 2001). Prior to in situ hybridizations, chromosome preparations were G banded and evaluated for ease of chromosome identification. Chromosome painting was performed, and images of fluorescence in situ hybridizations were captured and processed as previously described (Nash et al. 1998).

Results and Discussion

Chromosome Segment Homology by Chromosome Painting

For analysis of hybridizations using chromosome-specific painting probes, chromosomes are DAPI banded that produces chromosome banding patterns comparable with G-banding (Figure 1). We have shown previously that the domestic cat (FCA) genome is highly conserved relative to the ACK because many of the chromosomes are found conserved across several Carnivore families (Nash et al. 2001). By contrast, raccoon dog (NPR) chromosomes, as for most Canidae species, are highly rearranged relative to the cat (FCA) and by inference to the ACK (Nash et al. 2001). There exists 63 distinct synteny blocks that differentiate the FCA versus NPR karyotypes (Nash et al. 2001).

Chromosome painting probes for the domestic cat (FCA) and the raccoon dog (NPR) were hybridized to metaphase spreads of 3 Carnivore species: the ringtail (BAS)—family Procyonidae; dwarf mongoose (HPA)—family Herpestidae, and Malagasy civet (FFO)—family Viverridae. The hybridization signal of the cat (FCA) chromosome probe B1 completely paints a single dwarf mongoose (HPA) chromosome (Figure 1A). Full chromosome homology for cat B1 is also observed with the ringtail (BAS) and Malagasy civet (FFO) (Figure 1), whereas raccoon dog (NPR) chromosome 4 probe paints 5 different chromosome segments of the ringtail (BAS) (Figure 1B). Raccoon dog (NPR) chromosome 4 also painted multiple chromosome segments of dwarf mongoose (HPA) and Malagasy civet (FFO) (Figure 1 D,E). The chromosome homology segment patterns for each domestic cat (FCA) and raccoon dog (NPR) chromosome painting probes are summarized to the right and left, respectively, of the DAPI-banded metaphase chromosomes of each species (Figure 1 C–E). An examination of the hybridization patterns of cat (FCA) probes on 3 species (BAS, HPA, FFO) reveals that each cat (FCA) chromosome arm paints 1 or 2 complete chromosome arms in all 3 species. This is the pattern obtained when the chromosomes of species are highly conserved relative to each other. By contrast, multiple segments from different (up to 5) chromosome arms of these same 3 species are hybridized with raccoon dog (NPR) single chromosome painting probes because canid chromosomes are reshuffled considerably relative to the ancestral and conserved karyotype bearing Carnivore species (Figure 1) (Nash et al. 2001).

The reshuffled disposition of raccoon dog (NPR) chromosomes can be used to further resolve the degree of similarity or colinearity between the chromosomes of these conserved species. For example, chromosome BAS 5, HPA 5, and FFO 2 are homologous to cat (FCA) chromosome arm A1q (Figure 1 C–E). The sizes and order of raccoon dog (NPR) defined segments are the same in all 3 species (NPR-8, 14, 2, 6, 15) suggesting a common evolutionary origin of these segment orders. The DAPI-banding patterns of the 4 species (BAS, FCA, HPA, FFO) appear the same as

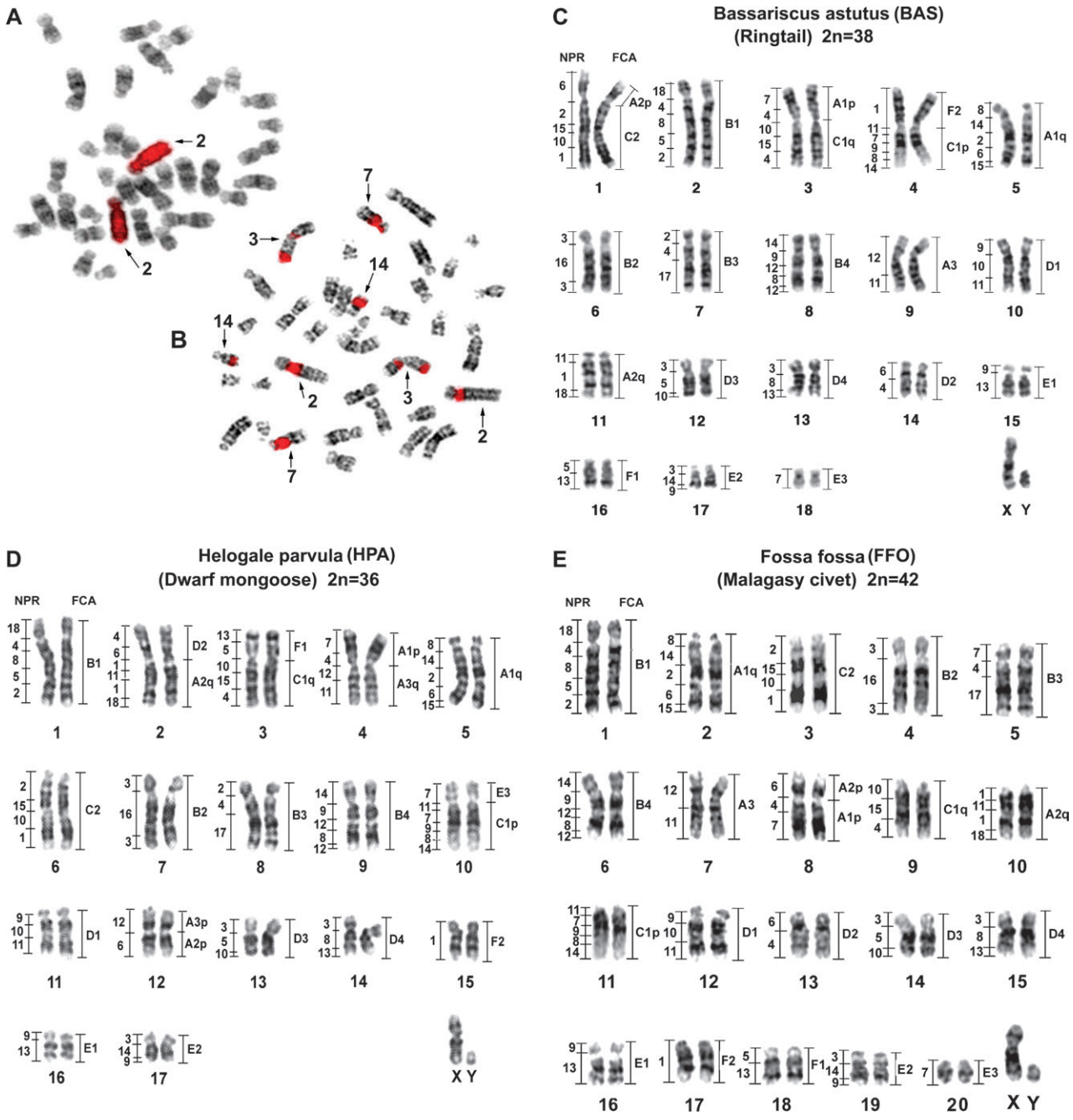

Figure 1. (**A**) Domestic cat (FCA) chromosome B1 painting probe hybridized to a dwarf mongoose (HPA) DAPI-banded metaphase spread. Cat chromosomes (FCA) typically have a one-for-one homology when painted on other chromosomally conserved Carnivore species such as dwarf mongoose (HPA) (Nash et al. 2001; Nie et al. 2002). (**B**) Raccoon dog (NPR) chromosome 4 painting probe hybridized to a ringtail (BAS) DAPI-banded metaphase spread. The rearranged mosaic raccoon dog chromosomes (NPR) typically hybridize to multiple regions of chromosomally conserved Carnivore species such as the ringtail (BAS) (Perelman et al. 2005). Hybridized chromosomes of the dwarf mongoose (HPA) and ringtail (BAS) are identified by arrows and numbers. (**C**) Karyotype of ringtail–BAS. Left bars summarize results of chromosome painting probes of raccoon dog-NPR on ringtail-BAS metaphase spreads; right bars summarize results of chromosome painting probes of domestic cat-FCA on ringtail-BAS metaphase spreads. (**C–E**) Chromosome paint results of raccoon dog (NPR) and cat chromosome-specific paints (FCA) are summarized adjacent to (C) ringtail (BAS), (D) dwarf mongoose (HPA), and (E) Malagasy civet (FFO) DAPI-banded karyotypes. Hybridization patterns of the raccoon dog (NPR) and cat (FCA) are shown to the left and right, respectively, of the chromosomes of each species. X chromosomes of each species are precise homologues as shown by chromosome painting.

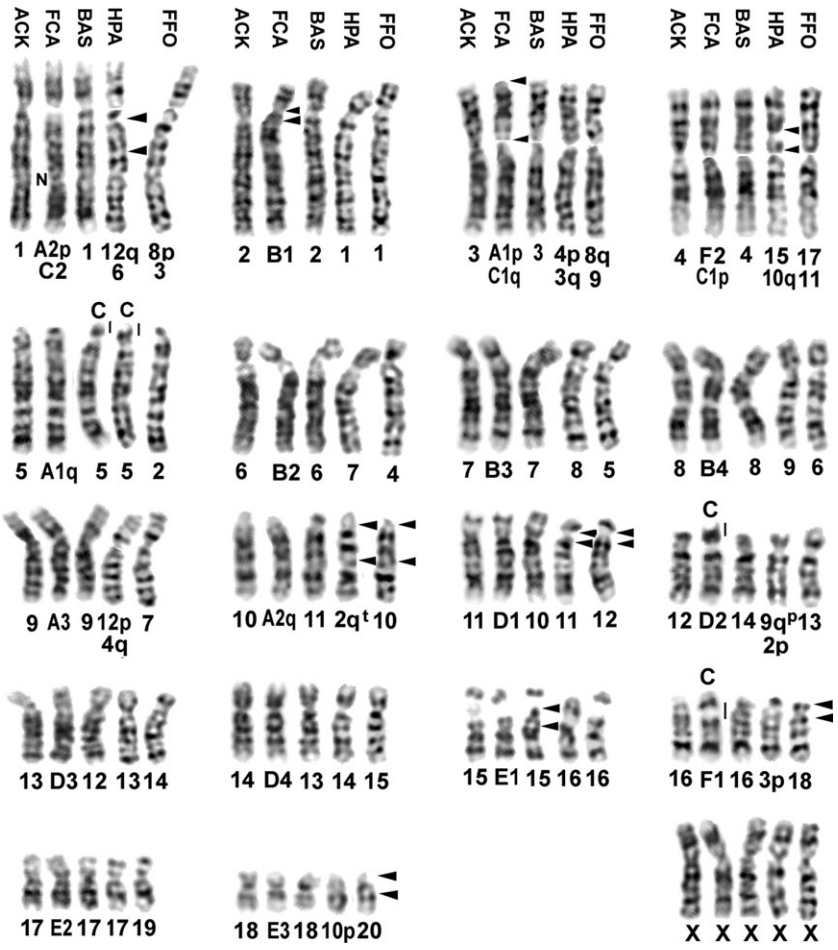

Figure 2. Proposed ACK compared with 4 species analyzed here. The ACK karyotype is a composite whose chromosomes are selected from the karyotypes of the domestic cat (FCA) and ringtail (BAS). The ACK autosomes are arranged and numbered according to their size. The homologous chromosome elements of the 4 karyotypes are identified under each chromosome. When 2 chromosomal elements, as a result of a centric fusion, are homologous to one ACK chromosome (e.g., FCA A2p and C2: row one, column one), the top and bottom cat chromosome element designations are homologous to the p and q arm of the ancestral chromosome, respectively. Chromosomes with an inversion relative to the ACK are indicated with arrowheads. The arrowheads next to these chromosomes identify breakpoints. A "C" at the top of a chromosome indicates a chromosome containing constitutive heterochromatin (C band). A vertical line identifies the location and element extent of the C band. *N* = position of a neocentromere.

well (Figure 2) where banded comparisons are of equally extended chromosomes for each species is presented.

Chromosomes BAS 11, HPA 2qter, and FFO 10 are homologous to cat chromosome arm FCA A2q (Figure 1 C–E). The homology segment sizes and linear order of raccoon dog (NPR) painting probes (1, 11, 1, 18) are the same in the dwarf mongoose (HPA) and Malagasy civet (FFO) but slightly different in the ringtail (BAS) (11, 1, 18). This difference is a result of one of 9 chromosomal inversions identified by arrowheads in Figure 2 (ACK 10).

The ACK Proposed

The first ACK was proposed by Duttrillaux and Couturier (1983). CAR as it was referred to was based primarily on the comparisons of R-banded karyotypes of Carnivores from 5 different families (Procyonidae, Mustelidae, Felidae, Phocidae, and Viverridae). It consisted of $2n = 42$ chromosomes and was similar to the ACK proposed by Murphy et al. (2001). The second proposed ACK called Z-CAR was based on banding comparisons of Wurster-Hill and Gray (1975) and on cat and seal FISH data (Frönicke et al. 1997). Z-CAR consists of $2n = 38$ chromosomes and is very similar to the ACK proposed in this study. Although previous investigators suggested that the relative lengths of chromosome arms and centromere positions of ACKs chromosomes are conserved, we demonstrate that the overall banding patterns are conserved as well. Furthermore, the current observation that *Nandinia*, a basal Feliformia species, has the ancestral bi-armed chromosomes ACK 3 and 4 (Figure 2) and is crucially important evidence that supports the ACK $2n = 38$ model.

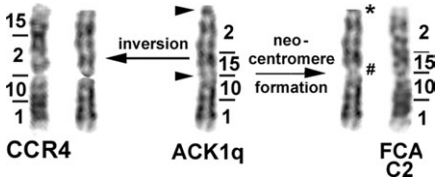

Figure 3. (**A**) Different evolutionary histories of chromosome arm ACK 1q in the domestic cat (FCA) and spotted hyena CCR. The numbers adjacent to the chromosome elements in this figure refer to fragments of raccoon dog (NPR) chromosomes that hybridize to these regions. In the cat (FCA), lineage acrocentric chromosome arm ACK 1q is rearranged into metacentric FCA C2 by inactivation of the terminal centromere (*) and activation of an interstitial centromere (#). The order of raccoon dog (NPR) chromosome-painting probes is the same for ACK 1q and FCA C2. The banding pattern is also the same. Hyena (CCR) chromosome 4 results from an inversion of ACK 1q whose breakpoints are identified with arrowheads. The order of painting probes 15–2 has been reversed in the hyena (CCR). The banding pattern has been reversed as well. In this figure, CCR 4 and FCA C2 are shown upside down. In spite of their independent origins, the centromeres of CCR 4 and FCA C2 are found at the same chromosome locus.

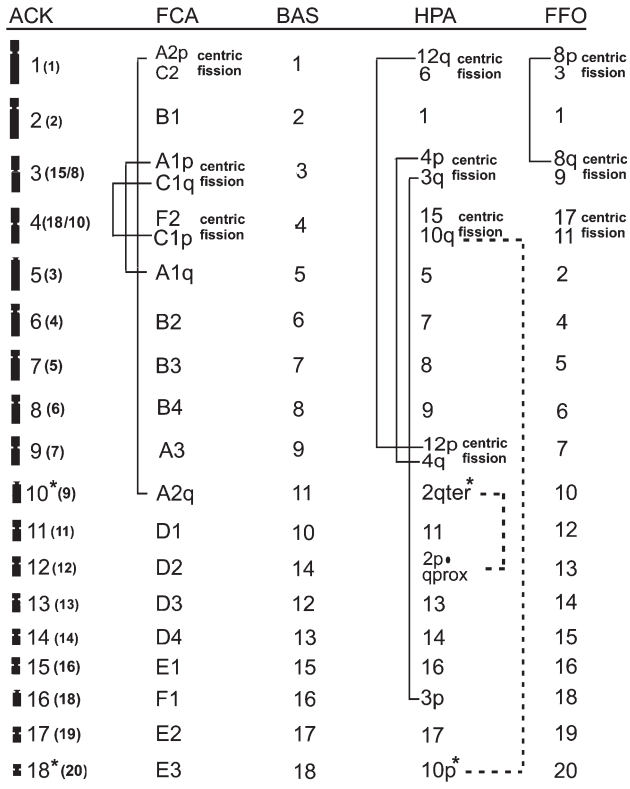

Figure 4. Centric fissions, centric fusions, and tandem fusions in the chromosomal evolution of 4 conserved carnivore karyotypes. The number, relative size, and centromere location for each autosomal ACK chromosome are shown in column 1. The chromosome numbers of the former proposed ACK are shown in parentheses (Murphy et al. 2001). The homologous chromosome elements for 4 species (FCA, BAS, HPA, and FFO) are also shown. Chromosome centric fissions relative to the ACK are indicated to the right of the 2 elements of each fission. For example, chromosome elements A2p and C2 of FCA are homologous to ACK 1p and 1q, respectively. Chromosome centric fusions relative to the ACK chromosomes are indicated by brackets to the left, in each column, and link the 2 fusion products. Each centric fusion was preceded by at least one prior centric fission of an ACK chromosome. Two chromosome tandem fusions in FFO are indicated by dotted brackets to the right and link the 2 fusion products. In tandem fusions, the centromere of once ancestral chromosome became inactivated. For example, in an HPA ancestor, bi-armed chromosome ACK 12 tandemly fused to acrocentric chromosome ACK 10. The centromere of chromosome ACK 10 became inactivated during the formation of HPA chromosome 2 (see Figure 6B). Each centric fusion was preceded by at least one prior centric fission of an ACK chromosome. Asterisks (*) mark which centromere of the tandemly fused chromosomes are inactivated both in the ACK and HPA homologues.

In the ACK $2n = 42$ proposal, metacentric chromosomes ACK 3 and 4 are represented as 4 acrocentric chromosomes (Duttrillaux and Couturier 1983; Murphy et al. 2001).

Arranging the chromosome arms of the 4 extant species (FCA, BAS, HPA, and FFO) relative to their ACK chromosome counterparts (Figure 2) implies the occurrence of derived centric fissions, fusions, and tandem fusions. The total number of euchromatic bands is the same in each species. Three examples of detectable chromosome expansion (C bands) are evident in homologues of ACK chromosome 5, 12, and 16, making the BAS 5, HPA 5 homologues, FCA-D2, and FCA-F1 slightly larger than the ancestral form (Figure 2). Otherwise, the overall banded phenotype of ancestral chromosomes is preserved in these modern karyotypes (Figure 2).

The "*N*" next to cat chromosome C2 in Figure 2 (FCA, row 1, column 1) identifies a new centromere location relative to its ACK position (neocentromere formation, Warburton 2004). Although this appears to be an inversion relative to ACK 1q, it actually results from inactivation of the ancestral centromere and activation of a new centrally positioned centromere. The absence of an inversion is demonstrated with raccoon dog (NPR) chromosome painting probes that hybridize in the same order (2, 15, 10, 1) on chromosomes, cat (FCA) C2, ringtail (BAS) 1q, dwarf mongoose (HPA) 6, and Malagasy civet (FFO) 3, which all have the same horizontal banding patterns as ACK 1q (Figure 1).

ACK 1q is also a metacentric chromosome in Hyaenidae karyotypes. In the spotted hyena, *Crocuta crocuta* (CCR), for example, chromosome CCR 4 is homologous to chromosome

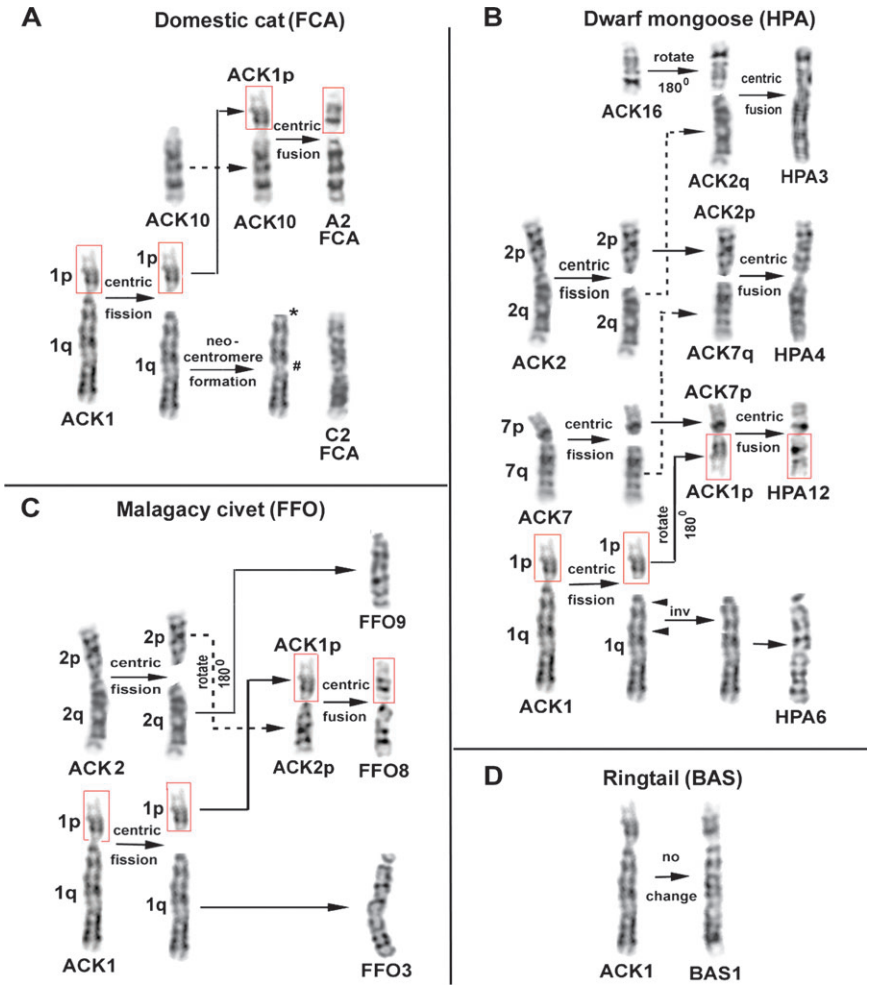

Figure 5. Panels (**A**, **B**, and **C**) follow the sequence of rearrangements of chromosome ACK 1 as it evolved in to its present horizontal banded disposition in 3 Feliformia species; the domestic cat (FCA), dwarf mongoose (HPA), and Malagasy civet (FFO). A centric fission of ACK 1 occurred in the ancestor of these 3 species. In each species, ACK 1p (highlighted with red boxes) subsequently fuses to a different ACK acrocentric chromosome whereas ACK 1q remains a separate chromosome. All the collateral chromosomes involved in subsequent rearrangements with ACK 1p are also shown (e.g., ACK 7, ACK 2, and ACK 16 in panel B). Neocentromere formation (A) is described in the text. In panel (A), * indicates centromere is inactive and # indicates centromere is active. Panel (D) shows that chromosomes ACK 1 and BAS 1 are identical at the 400-band level of resolution.

FCA C2. Regional canid chromosome probes show that the centromere of FCA C2 and CCR 4 are located at the same relative position and yet they have independent origins (Figure 3). In the hyena lineage, a pericentric inversion changed ACK 1q into the metacentric CCR 4 (Figure 3). FCA C2 and CCR 4 are signature chromosomes of their respective families found in all felids and hyaenids but not in species of any other Carnivora families. The independent origins of FCA C2 and CCR 4 are consistent with the phylogenetic distance between Felidae and Hyaenidae.

Figure 4 is a compilation of a summary of centric fissions, centric fusions, and tandem fusions found in the domestic cat (FCA), ringtail (BAS), dwarf mongoose (HPA), and Malagasy civet (FFO), respectively, relative to the ACK. The relative size and centromere location of ACK chromosomes are shown in the first column. The homologous chromosomes or chromosome arms of each species are listed in the remaining 4 columns. Centric fissions of ancestral chromosomes produce 2 new chromosome elements. For example, bi-armed ACK chromosomes 1, 3, and 4 are separated by centric fissions in the 3 Feliformia species FCA, HPA, and FFO (Figure 4). To visualize this process, see Figure 3, which illustrates the fate of ACK 1 in the 4 species studied here. In the ancestors of the 3 Feliformia species, domestic cat (FCA) dwarf mongoose (HPA) and Malagasy civet (FFO), chromosome ACK 1 underwent a centric fission. ACK 1p subsequently

fused to a different chromosome arm in each species whereas ACK 1q remained a separate chromosome. Acrocentric ACK chromosome 10 and ACK 1p fuse at their centromeres, resulting in the formation of cat (FCA) chromosome A2 (Figure 5A). The original centromere of ACK chromosome arm 1q was inactivated and a new centrally located centromere activated to form metacentric chromosome FCA C2 (Figure 5A).

The fate of chromosome ACK 1 in the dwarf mongoose (HPA) lineage is shown in Figure 5B. Three centric fissions and 3 subsequent centric fusions of ACK chromosome elements resulted in dwarf mongoose (HPA) chromosomes 3, 4, and 12. ACK 1q became dwarf mongoose (HPA) chromosome 6. A paracentric inversion in HPA-6 was resolved with G-banding but was too small to be identified with raccoon dog (NPR) regional painting probes.

In the Malagasy civet (FFO) lineage, the centric fission of chromosomes ACK 1 and 2, followed by the centric fusion of ACK 1p and ACK 2p resulted in the Malagasy civet (FFO) chromosomes 3, 8, and 9 (Figure 5C). Chromosome ACK 1 was unchanged in the ringtail (BAS) as it was in the other chromosomally conserved Caniformia species (Figure 5D). The banding pattern proposed for the ancestral chromosomes can be traced in the transitional and final chromosomes of these 4 species (FCA, BAS, HPA, and FFO).

Two independent tandem fusions of ACK chromosomes occurred in the dwarf mongoose (HPA) lineage and are presented in Figure 6. In Figure 6A, the centromere region of acrocentric chromosome ACK 10 tandemly fused to the telomere region of the p arm of chromosome ACK 12. ACK 12 evolved into the p arm and the proximal q arm of dwarf mongoose (HPA) chromosome 2 as a result of the centromere inactivation of acrocentric chromosome ACK 10. A paracentric inversion of ACK 10 in the dwarf mongoose (HPA) lineage (arrowheads in Figure 6A) was revealed with raccoon dog (NPR) regional probes. This inversion gave rise to the banding pattern seen in dwarf mongoose (HPA) chromosome 2qter. Figure 6B presents the tandem fusion of ACK 4q and ACK 18. After the centric fission of ACK 4, the centromere region of ACK 4q fused to the telomere region of the p arm of chromosome ACK 18. Inactivation of the centromere of ACK 18 led to dwarf mongoose (HPA) chromosome 10. ACK 4p underwent a pericentric inversion to become dwarf mongoose (HPA) chromosome 15.

G-Banding Conservation among Carnivore Chromosomes

Figures 2, 5 and 6 illustrate how chromosome regional painting probes hybridized to metaphase spread with equally extended banded chromosomes can provide a detailed pictorial of individual and comparative chromosome evolution. Each chromosome can be followed band for band to reveal centric fissions and fusions, tandem fusions with position of attendant centromere activations and inactivations, inversions with their breakpoints, and additions of heterochromatin.

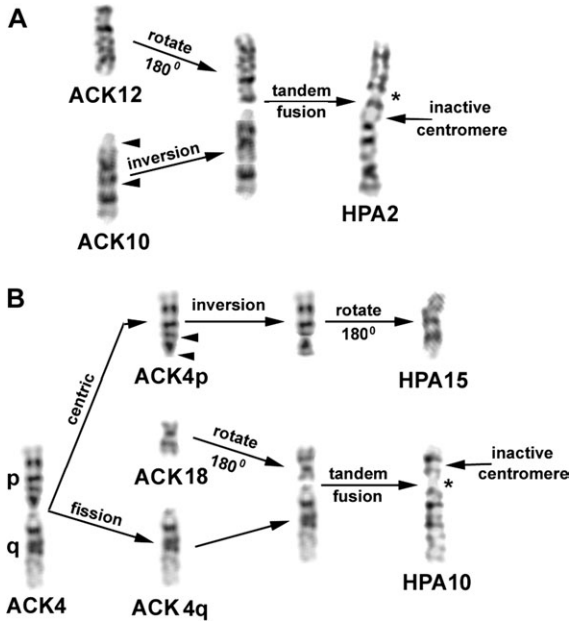

Figure 6. Tandem fusions of ACK chromosomes in the dwarf mongoose karyotype (HPA). (**A**) The centromere of acrocentric chromosome ACK 10 tandemly fuses to the telomere end of the short arm of ACK 12. The centromere of ACK 10 is inactivated in the tandem fusion process that gives rise to chromosome HPA 2. The inversion in ACK 10 probably occurs before it is tandemly fused to ACK 12. (**B**) ACK 4 splits into acrocentric chromosomes as the result of a centric fission. An inversion in ACK 4p leads to HPA 15. The centromere of acrocentric chromosome arm of the ACK 4q tandemly fuses to the telomere end of the short arm of ACK 18. The centromere of the bi-armed chromosome ACK 18 is inactivated during the tandem fusion process that gives rise to chromosome HPA 10. * indicates active centromere.

Reconstructing a banded karyotype is only possible if it can be demonstrated that the banding patterns seen in the chromosomes of modern species are derived one-for-one from a common ancestor that lived some 60 million years ago. A two-tier approach has been adopted to reveal the degree of banding conservation in Carnivore chromosomes. First, domestic cat painting probes were used in cross-species hybridizations. Most domestic cat (FCA) chromosomes are exact homologues of the putative ACK chromosomes and, therefore, serve as excellent probes for identifying ancestral chromosome homologues in other Carnivore species (Nash et al. 2001). Cat-based cross-species hybridizations demonstrate that centric fissions and fusions play a dominant role in changing the chromosome number and morphology of modern species karyotypes (Nash et al. 1998, 2001). No reciprocal translocations have been observed in the karyotypes of modern Carnivore species. Second, raccoon dog (NPR) chromosome painting probes were used in cross-species hybridizations. Canid chromosomes are mosaics of ancestral chromosome arm fragments and can, therefore, be utilized as "regional chromosome

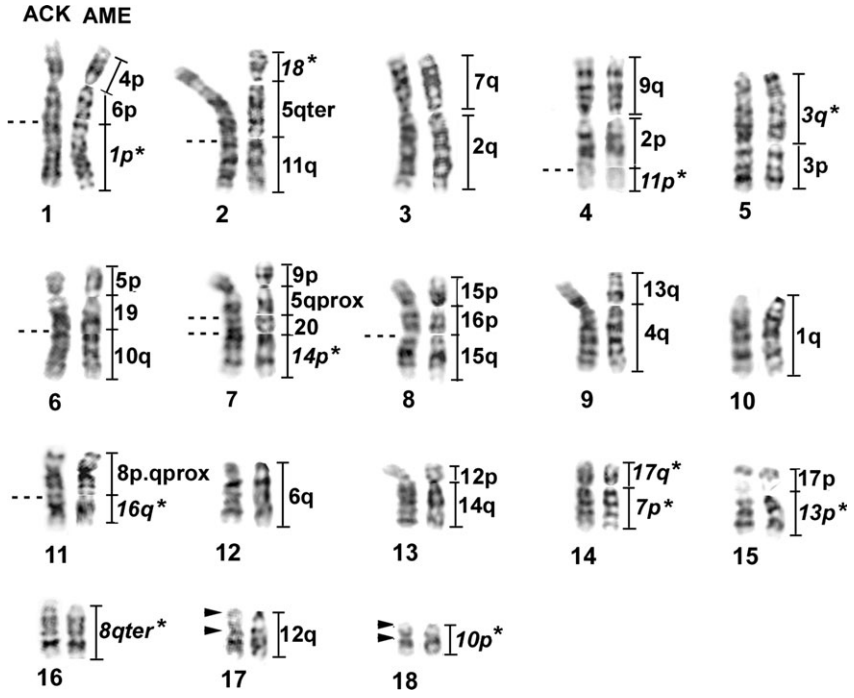

Figure 7. DAPI-banded homologous chromosome elements of the giant panda AME to the right are compared with the putative DAPI-banded ACK chromosomes (left). The number–letter designations to the right identify giant panda (AME) chromosome elements. Banded comparisons are based on giant panda chromosome-specific painting probes hybridized to ACK chromosome homologues as they exist in modern species (Nash et al. 1998). Note the degree to which the banding patterns are preserved even in the relatively rearranged ursid karyotype. Arrowheads indicate inversion breakpoints. --- indicates ACK chromosome arm breakpoints that are common to all Ursidae species. * indicates AME chromosome elements inverted.

segment painting probes" to identify inversions. C-banding was performed to identify saltatory additions of constitutive heterochromatin.

Including the 3 species in this report (BAS, HPA, FFO), cat painting probes (FCA) have been hybridized to species of the Canidae (3 spp.), Ursidae (3 spp.), Procyonidae (2 spp.), Mustelidae (6 spp.), Phocidae (1 spp.), Viverridae (2 spp.), Herpestidae (1 spp.), and Hyaenidae (1 spp.) (Frönicke et al. 1997; Hameister et al. 1997; Nash et al. 1998, 2001; Cavagna et al. 2000; Yang et al. 2000; Nie et al. 2002; Tian et al. 2004). Domestic dog (*Canis familiaris*, CFA) or raccoon dog (NPR) chromosome painting probes have been hybridized to Canidae (5 spp.), Ursidae (3 spp.), Procyonidae (1 spp.), Mustelidae (1 spp.), Viverridae (2 spp.), Herpestidae (1 spp.), Hyaenidae (1 spp.), and Felidae (3 spp.) (Nash et al. 1998, 2001; Yang et al. 1999; Graphodatsky, Yang, O'Brien, et al. 2000; Graphodatsky, Yang, Serdukova, et al. 2000; Yang et al. 2000; Graphodatsky et al. 2001; Tian et al. 2004; Perelman et al. 2005).

Six species' chromosome comparisons in Figure 2 have invariant banding patterns in all 4 species (ACK 6, 7, 8, 13, 14, and 17). Except for ACK 8, these chromosome homologues are also invariant in *Martes foina* and *Meles meles* (Mustelidae), *Phoca vitulina* (Phocidae), and CCR (Hyaenidae) (Frönicke et al. 1997; Nie et al. 2002; Perelman et al. 2005). These chromosomes, identical by Zoo-FISH and DAPI-/G-banding criteria, seen in diverse Feliformia and Caniformia families, are considered ancestral.

When defining an ancestral version, choosing any chromosomes from these sets would be equally accurate

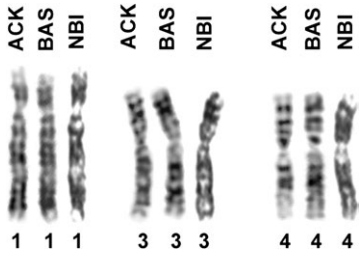

Figure 8. Three proposed ACK chromosomes that occur in ringtail (BAS 1, 2, and 3) and the African palm civet (NBI 1, 2, and 3). The BAS chromosomes are DAPI banded whereas the NBI chromosomes are G banded (from Wurster-Hill and Gray 1975). These 3 bi-armed ACK chromosomes are found in all conserved families of the Caniformia and in Nandiniidae but have undergone centric fissions in other Feliformia families.

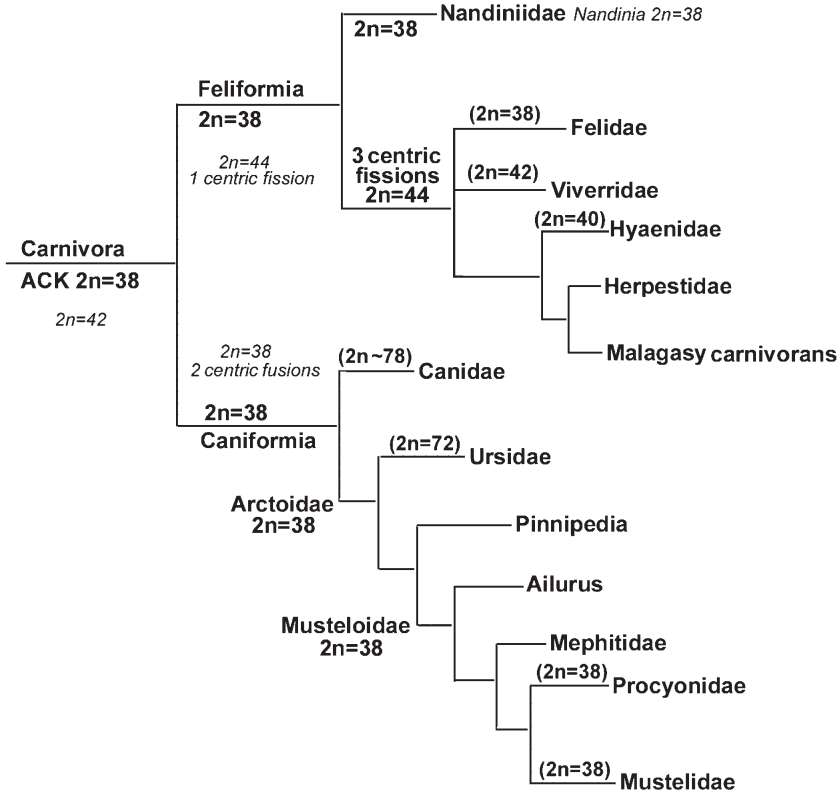

Figure 9. A schematic cladogram of the major evolutionary relationships of the Carnivora modified from Flynn et al. (2005). Proposed 2*n* chromosome numbers and rearrangements at basal branch points are shown. In order to reconcile the observation that *Nandinia* (NBI) has 3 chromosomes that were previously thought to occur only in the Caniformia species, the 2*n* number of the ACK was changed from 42 to 38. Chromosome numbers and rearrangements are shown for ACK 2*n* = 38 (bold face) and ACK 2*n* = 42 (italics). The former ACK (2*n* = 42) proposed a chromosome centric fission after the split of the Feliformia (see Figure 5) and 2 chromosome centric fusions after the split of the Caniformia (see Figure 4; column 1, rows 3 and 4, in parentheses). It does not explain the 3 Caniformia "signature" chromosomes in *Nandinia* (NBI). The new ACK (2*n* = 38) shows no change of chromosome number in the early Feliformia and Caniformia (2*n* = 38). It postulates that 3 chromosome centric fissions of ACK 1, 3, and 4 occurred after the split separating Nandiniidae but before the split of the other Feliformia families (2*n* = 44). The ancestral chromosome number (2*n*) of each family is indicated where supporting data are available. The 2*n* ancestral chromosome number for the Ursidae and Canidae have been previously defined (Nash et al. 1998, 2001).

or valid. The domestic cat (FCA) and ringtail (BAS) chromosomes are mostly ancestral; therefore, the ACK karyotype shown in Figure 2 is a composite of domestic cat (FCA) and ringtail (BAS) chromosomes. If centric fissions are reversed, as is the case in Figure 2, then the ACK 9 homologue set is invariant as well. In the absence of the heterochromatin in the short arms of homologue sets ACK 5 and 12, these chromosomes are also invariant. It is clear from Figure 2 that inversions large enough to noticeably alter chromosome banding patterns are uncommon in these species. When inversions do occur, the chromosome banding pattern present in both Feliformia and Caniformia species is considered ancestral. Consider homologue set ACK 10 in Figure 2, where cat (FCA, Feliformia) and ringtail (BAS, Caniformia) chromosome elements have the ancestral banding pattern while the inversion found in the dwarf mongoose (HPA) and Malagasy civet (FFO) is derived. This conclusion is supported by cross-species hybridizations of cat (FCA) and domestic dog (CFA) chromosome painting probes on the hyena (CCR), Malayan sun bear (*Helarctos malayanus*), and giant panda (AME, Caniformia) (Nash et al. 1998; Tian et al. 2004; Perelman et al. 2005). The order of hybridized regional dog probes to the homologues of ACK 10 in these 3 species is the same as that found in the cat (FCA) and ringtail (BAS). When the inversions identified in Figure 2 are reversed, the ancestral banding patterns are restored, strongly supporting the conclusion that ancestral chromosome banding patterns are conserved.

Taken together, the information from Figure 2 and Figure 4 provides a detailed picture of chromosome evolution in these species. Based on the molecular and classical cytogenetic techniques employed here, the ringtail

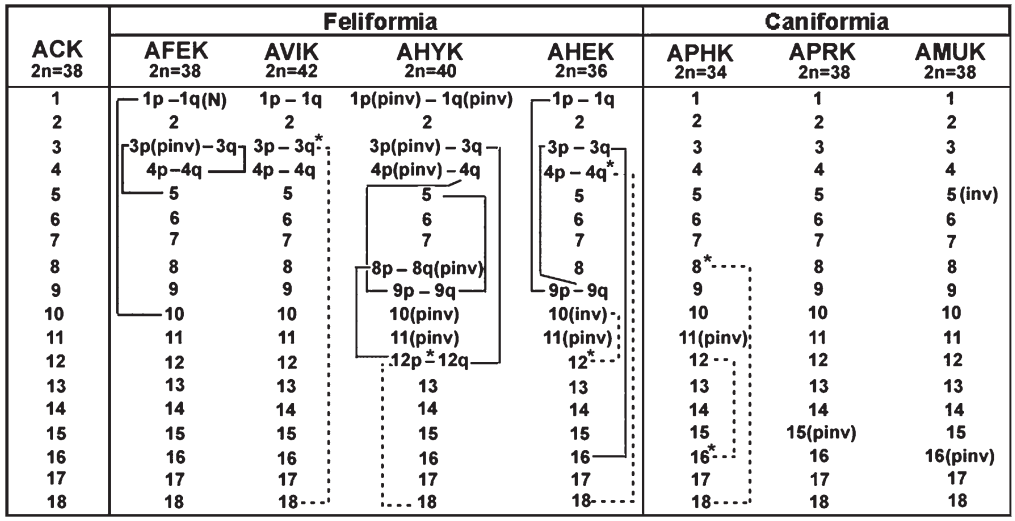

Figure 10. Putative ancestral karyotypes of 7 Carnivora families compared with the ACK. ACK, ancestral Felidae karyotype (AFEK), ancestral Viverridae karyotype (AVIK), ancestral Hyaenidae karyotype (AHYK), ancestral Herpestidae karyotype (AHEK), ancestral Procyonidae karyotype (APRK), ancestral Mustelidae karyotype (AMUK). Relative to the ACK chromosomes, centric fissions are indicated by a dash (–) between p and q arms. As in Figure 1, solid line brackets indicate centric fusions. Dotted line brackets indicate tandem fusions. In the Hyaenidae, ACK chromosome arms 8p and 12p are joined by a centric fusion and ACK 18 is tandemly joined to the telomere end of the ACK 12p forming a tripartate chromosome. There are 4 species in the family Hyaenidae. The 2 species analyzed cytogenetically, the spotted hyena (CCR) and the brown hyena (*Parahyaena brunnea*) both have the same karyotype (Nash WGN, personal observation). pinv = pericentric inversion, inv = paracentric inversion, * = centromere that is active after tandem fusion. The Zoo-FISH references for this figure appear in the third heading of the Results and Discussion. DAPI-/G-band karyotypes analyzed include Felidae (Wurster-Hill and Gray 1975; Wurster-Hill and Centerwall 1982), Viverridae (Wurster-Hill and Gray 1975; Graphodatsky 2006), Hyaenidae (Nash 2006; Graphodatsky 2006), Herpestidae (Wurster-Hill and Gray 1975; Yang and Li 2006), Phocidae (Arnason 1974a,b; Arnason 1977), Procyonidae (Wurster-Hill and Gray 1975; Stanyon et al. 1993; Stanyon 2006), and Mustelidae (Wurster-Hill and Centerwall 1982; Graphodatsky 2006). The sex chromosomes in these karyotypes are not shown.

(BAS) has the most conserved karyotype of any modern Carnivore species examined (Figure 4). It differs from the ACK by the addition of heterochromatic short arms on ACK 5 and one pericentric inversion in chromosome ACK 15 (Figure 2). For species with conserved rearranged karyotypes, the degree of fine detail preserved in the chromosome banding patterns is remarkable. Based on previous hybridization results, the DAPI-banded giant panda chromosomes (AME) are compared with the ACK in Figure 7 (Nash et al. 1998).

In conserved carnivore species (i.e., all except Ursidae and Canidae), ancestral chromosome arm organization is seldom affected, save for occasional para- and pericentric inversions. In the Ursidae, but far more so in the Canidae, ancestral chromosome arms are fragmented by repeated cycles of pericentric inversions and centric fissions then reassembled into mosaic blocks by tandem fusions and metacentric to acrocentric inversions (Nash et al. 1998, 2001). Nonetheless, all DAPI/G bands at the microscopic level of resolution are conserved and recognizable by their individual band morphologies. The extreme conservation of individual chromosome band morphology suggests that these structures are fundamental units of gene organization.

Basal Chromosome Rearrangements

Three proposed ACK homologues (ACK 1, 3, and 4) occur in most Caniformia families as well as in the Feliformia basal family Nandiniidae, (African palm civet, NBI) (Figure 8). The presence of these intact metacentric homologues in species of both Caniformia and Feliformia lineages suggests that the bi-armed representations were ancestral, hence its inclusion in the proposed ACK (Figures 2 and 8). These chromosomes subsequently diverged into acrocentric derivatives after the divergence of Nandiniidae from the remaining Feliformia species. Finding chromosomes ACK 1, 3, or 4 in any modern Feliformia species other than *Nandinia* would invalidate this interpretation. Wurster-Hill and Gray (1975) reported the presence of CAR 22 (ACK 3) in 2 mongoose species, *Bdeogale sp.* and *Atilax paludinosus*. The chromosomes they identified as CAR 22 in both species are actually homologous to chromosome 4 of the dwarf mongoose, which our painting data demonstrate is homologous to ACK 3p fused to ACK 9q at their centromeres. They also reported that CAR 25 (ACK 4) is found in the civet *Viverricula indica*, but this chromosome was also misidentified and is actually ACK 10 and 16 fused at their centromeres. Therefore, *Nandinia* is still the only

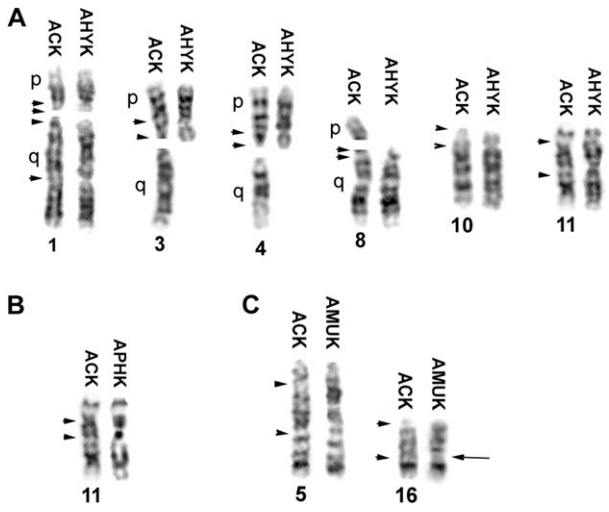

Figure 11. (**A**) Chromosomes of the putative ancestral Hyaenidae family karyotype (AHYK) that differ from their ACK chromosome homologues by a single pericentric inversion. In each comparison, ACK chromosomes are shown to the left with inversion breakpoints indicated by arrowheads. The ancestral hyaenid chromosomes are shown to the right and inverted relative to the ACK chromosome homologues. (**B**) The single pericentric inversion found in the ancestral Phocidae family karyotype (APHK). (**C**) The single pericentric inversion found in the Mustelidae family karyotype (AMUK). The arrow in (D) identifies the centromere. B, C, and D in combination with Figure 10 can be used to reconstruct the ancestral family karyotypes of Hyaenidae, Phocidae, and Mustelidae.

Feliform species found to date with chromosomes ACK 1, 3, and 4.

In Figure 9, we present a recent Carnivora family-level phylogeny (after Flynn et al. 2005) including chromosome numbers of stem species, ancestral mode chromosome numbers plus, and postulated fission/fusion events plus chromosomes exchanged. The Malagasy civet (FFO) shares 2 inversions in common with the dwarf mongoose (HPA) and other herpestid species. In contrast, these inversions are not found in species of the Viverridae. This is consistent with the molecular phylogeny that suggests that the Malagasy civet (FFO) is more closely allied to the Herpestidae rather than the Viverridae.

Putative Carnivore Family Ancestral Karyotypes

If Zoo-FISH and chromosome banding, data are available for a significant number of modern species of a family, then the chromosome number and chromosome disposition of the ancestral karyotype of that family can be determined. With rare exception, the only rearrangements that change chromosome number in Carnivore families with conserved karyotypes are centric fissions, centric fusions, and tandem fusions. Therefore, to establish the ancestral chromosome number of a family, one need only determine the number of ACK chromosomes involved in these 3 rearrangements that are common to all family members. As an example, refer to the centric fissions and centric and tandem fusions of the dwarf mongoose (HPA) in Figure 4, which as it turns out are also common to other herpestid species (Wurster-Hill and Gray 1975; Yang and Nie 2006). The ACK 2*n* chromosome number is 38; therefore, the ancestral chromosome number for the family Herpestidae is 38 plus 4 centric fissions (+8), 3 centric fusions (−6), and 2 tandem fusions (−4) = 36. Based on all current Zoo-FISH and banded chromosome data available, the putative ancestral chromosome number for 7 Carnivore families is shown in Figure 10.

One can also reconstruct the longitudinal banded phenotype of each chromosome in a family ancestral karyotype. Banded chromosomes are visually so rich in information that exact duplicates never evolve independently of one another. Therefore, if a modern species has an ancestral chromosome in its karyotype, its family ancestor had this chromosome as well. In the Procyonidae, for example, 17 of 18 autosomal chromosomes are the same as their ACK homologues in the karyotypes of the ringtail (BAS), raccoon (*Procyon lotor*) (Stanyon et al. 1993), and the kinkajou (*Potos flavus*) (Stanyon 2006). All procyonid species have a pericentric inversion in the homologue of ACK chromosome 15, and, therefore, this chromosome variant is ancestral for the family. The homologue of ACK chromosome 15 remains unchanged in the Mustelidae, and this requires that the ancestors at all earlier Caniformia branch points (Arctoidae and Musteloidae) had the ACK (see Figure 9).

Figure 10 compares the ancestral chromosomes of 7 Carnivore families to their ACK chromosome homologues. Symbols for centric fissions, centric fusions, and tandem fusions are described in the figure legend. All the breakpoints for the paracentric and pericentric inversions listed in Figure 10 can be found in Figure 2 and Figure 11A–C, and using this information, one can individually reconstruct the chromosomally banded ancestral karyotype for all 7 families. The ancestral Mustelidae karyotype (Figure 10) is nearly identical to the consensus *Martes*-like karyotype reported previously by Graphodatsky et al. (2002) and both closely resemble the ACK.

Rate of Karyotypic Evolution in 3 Carnivore Species with Conserved Karyotypes

The resolution of karyotypic evolution at the microscopic level is informed primarily by the extension and quality of the chromosomes to be banded and hybridized. It is with this in mind that the karyotypes of the ringtail, dwarf mongoose, and Malagasy civet were evaluated. The ringtail karyotype as presented in Figure 2 is very similar to the ACK. It differs by the addition of a small amount of heterochromatin at the centromere of BAS chromosome 5 and a small pericentric inversion in BAS chromosome 15.

ACK chromosomes 1, 3, and 4 are intact in *Nandinia* but separated by centric fissions in all other Feliforms. The

branch points separating the *Nandinia* lineage from other Feliformia occurred approximately 50 mya. The only karyotypic changes in the Malagasy civet because the separation are 4 inversions and 1 centric fusion. Karyotypic changes in the dwarf mongoose include 4 inversions, 1 centric fission, 3 centric fusions, and 2 tandem fusions. By contrast, the domestic dog CFA karyotype differs from the ACK by 38 centric fissions and 23 centric fusions (Nash et al. 2001). As demonstrated in this investigation, the proposed high-resolution chromosome analysis of longitudinally banded ACK chromosomes has been an important tool for understanding the relationships of the chromosomes of different Carnivore species. It was the impetus for reexamining previously published karyotypes that led to the identification of chromosomes ACK 1, 3, and 4 in the African palm civet (NBI). This observation in conjunction with the basal position of Nandinia (NBI) relative to other Feliformia species (Flynn et al. 2005) supports the proposed ACK $2n = 38$. The postulated ACK gains support from painting, segment order homology, G-/DAPI-banding, and evolutionary history, which together provide a robust view of genome evolution in the Carnivora order.

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